Evaluating use of AAV in reprogramming glial cells into neurons post stroke

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Chapter 1. Introduction

Stroke is a leading cause of death, dementia, and long-term disability in the United States.[1] Despite being a preventable disease, stroke affects approximately 800,000 people every year.[1] Risk factors include elevated blood pressure, sedentary lifestyle, and high cholesterol. High blood pressure alone is associated with 84% of reported cases.[2] Stroke is characterized by a loss of blood flow or damage to the brain which results in neuron death, inflammation, and an immune response in the central nervous system (CNS). There are two major types of strokes, hemorrhagic and ischemic. Hemorrhagic stroke occurs when vessels weaken and rupture causing blood to flow into surrounding tissue and accounts for 40% of death.[1] Current standard of care works to stop bleeding, reduce brain swelling, and decrease high blood pressure. Major surgical interventions reduce intracranial pressure, restrict blood flow to the aneurysm, or physically seal off the aneurysm itself. Ischemic stroke occurs when blood vessels become obstructed by a clot in the central nervous system and accounts for roughly 85% of reported cases.[1] While endovascular surgical options to remove clots exist, IV administration of tissue Plasminogen activator (tPA) has been the gold standard and the only FDA approved treatment for ischemic stroke since 1996. tPA is a "clot busting" serine protease that must be administered within 4.5 hours after stroke onset.[3]

Early action is essential for stroke because every minute counts. Ongoing neuron death leads to significant brain damage. Researchers estimate that each minute after stroke results in the death of 1.9 million neurons, 14 billion synapses, and 7.5 miles of myelinated fibers.[4] Furthermore, every 30 minute delay before treatment leads to a 20% reduction in regaining function later on.[5] Seeking medical attention within the treatment

window has proven to be difficult in the United States. 3 to 5% of stroke patients receive tPA and less than 1% receive surgery within the 4.5 hour therapeutic window.[6] A lack of public awareness on how to spot and respond to early stroke symptoms further impedes a rapid response to care. A 2005 report by the CDC found that 38% of Americans could recognize the five major symptoms and proceed to call 911.[7] Furthermore, stroke centers remain out of reach for many Americans. Roughly 20% of individuals live more than 1 hr away from tPA equipped centers and 50% are 1 hr away from stroke surgery center.[9]

The low treatment rate leaves people with a range of disabilities that negatively impact their quality of life and requires assistance to manage. 70% of stroke victims experience upper and/or lower limb weakness and roughly 50% have trouble with either swallowing, incontinence or slurred speech.[10] Early on care works to prevent future strokes, prevent further complications, and minimize known risk factors. The transition into long term rehabilitation focuses on physical therapy to regain mobility, strength, coordination and range of motion in their limbs as well as cognitive therapy to help overcome memory and problem solving deficits. The difficulty for the six million American stroke survivors working towards functional recovery can be partially attributed to the limited regenerative capacity of the CNS after injury.

Pathology of Stroke

The CNS is comprised of glia, endothelial cells, and neurons. Glia play a supportive role and make up 91.7% and 55.4% of cells in the cerebral cortex of the human and mouse, respectively.[12] Glia include oligodendrocytes, microglia, and astrocytes. Oligodendrocytes produce and maintain myelin sheath that surround neuronal axons. Microglia scavenge throughout the CNS looking for damaged tissue or cellular

debris. They are derived from bone marrow macrophages that migrated into CNS during early stages of development. Astrocytes serve as an interface between the blood vessels and neurons to facilitate the transfer of glucose and waste. After injury to the central nervous system, the intricate relationship between neurons and glial goes into disarray and works to impede neuronal regeneration for the reasons discussed below.

Astrocytes have a dualistic response to CNS injury. Under normal physiological conditions, astrocytes play a regulatory role by maintaining and regulating neuronal synapses that neurons could not do alone. They maintain equilibrium through uptake of ions from the extracellular space and uptake of excess neurotransmitters from the synaptic cleft during neurotransmission.[15] Some researchers hypothesize that astrocytes support the metabolism of neurons in a model called the astrocyte-neuron lactate shuttle (ANLS).[16] In this model, glial cells tie anaerobic glycolysis to the uptake of K^+/Na^+ and the neurotransmitter, glutamate. Astrocytes uptake glucose from the bloodstream and transport it in the form of lactate to neurons. Then neurons produce energy in the form of ATP from lactate. This relationship between astrocytes and neurons breaks down and compounds injury to the CNS. As disease and damage progresses, astrocytes develop difficulties with glutamate uptake which leads to hyper stimulation and cell death for synapsing neurons.

The formation of the glial scar at the lesion site is another hallmark of CNS injury. Glial cells bordering the lesion begin to proliferate. They upregulate expression of filament proteins such as glial fibrillary acidic protein (GFAP), nestin, and vimentin. Furthermore, astrocytes upregulate inhibitory chondroitin sulfate proteoglycans (CSPGs) that contribute to the formation of a physical barrier and play a key role in restricting

axonal growth.[17] Together these factors help astrocytes remodel, isolate, and protect the lesion from further damage through the formation of the glial scar and closure of the blood brain barrier. More specifically, these reactive astrocytes develop a tight mesh network in a perpendicular orientation that oppose the normal longitudinal growth of axons. Under these conditions, the regenerative capacity of neurons is remarkably limited.

For many years, researchers believed that secretion of CSPGs by reactive astrocytes were primarily responsible for inhibition of new neuronal growth post-injury. However, ablation of reactive gliosis and complete inhibition of the glial scar formation by knockdown of STAT3 did not allow for spontaneous axonal growth.[18] Instead, total levels of extracellular of CSPGs were unaffected and were found to be secreted from GFAP negative sources such as oligodendrocytes and NG2+ cells in the CNS. This finding suggests that reactive astrocytes are not the only obstacle to neuronal regeneration and that we should consider the contributions of other cell populations.

Resident microglia respond in the first few minutes after injury. Under normal conditions, microglia form tight junctions at neuronal synapses and eliminate synaptic debris through phagocytosis. The loss of neuronal-glial interactions and secretion of their damage-associated molecules activate microglia to become pro-inflammatory. Upon activation, microglia recruit circulating leukocytes, initiate a strong inflammatory response around the lesion, and increase their phagocytic activity to clear up dead tissue. Microglia release metalloproteases and generate radical oxygen species like superoxide and hydrogen peroxide which contribute to the degradation of the blood brain barrier and neuronal destruction. Microglia activation has been correlated with injury in macrophage

co-cultures with either oligendrocytes or with astrocytes. When macrophage activation was inhibited in mouse models, infarct size was reduced.[14] However, there was no change in infarct size for mice that lacked macrophages circulating in the bloodstream. It is unclear if resident microglia or infiltrating macrophages are the primary contributors to blood brain injury.[11]

In Vivo Reprogramming

Although, adult neurogenesis does occur in the subgranular zone within the hippocampus as well as in the olfactory bulb. These endogenous sources of neurons are insufficient to repair the CNS.[11] In the past decade, researchers have looked away from spontaneous neurogenesis and instead towards working on directly reprogramming endogenous glial cells to replace the neurons lost during injury. As a potential therapy, *in vivo* reprogramming is considered advantageous compared to cell transplantation because it avoids immunorejection concerns, targets already proliferating cells, and the timed targeting could replace cells known to secrete inhibitory factors with new neurons.[19] Unfortunately, development of high efficiency reprogramming factors *in vitro* have not translated well to *in vivo* conditions. Efficiency is remarkably reduced *in vivo* compared to cell culture. Comparison of results among studies can be difficult without standardized conditions. Reprogramming efficiencies can vary from study to study depending on regional-specific differences in glial plasticity, vector design, and means of evaluating neuron conversion efficiency.[21,22,23] Maximizing reprogramming efficiency is paramount if we are to develop a gene therapy but this path remains difficult because a single glial cell can only give rise to one non-dividing neuron.

In vivo reprogramming of astrocytes and NG2-glia into neurons have been demonstrated in CNS injury mouse models.[25] However, the most effective endogenous source of cells is not currently known. Cell type specific transcriptional differences may be the ultimate determinant when finding the optimal source.[24] Differences in amount of heterochromatin may limit access to critical neurogenic loci and create an overall transcriptionally repressed state. Activity of terminal selector gene could help drive neuronal fate commitment by coordinating activation of identity specific genes while simultaneously repressing non-identity gene or vice versa. Lastly, starting with a cell population whose transcriptome profile more closely resembles neurons is believed to have better efficacy compared to ones that are farther removed.

Furthermore, multiple pro-neural transcription factors have been used to reprogram non-neuronal cells such as Ngn2, Ascl1, Sox2, and NeuroD1.[22] Which of these is most effective is also unknown. However, they are known to be involved at different time points during neurogenesis and are thought to play a role in chromatin remodeling to create a more transcriptionally permissive state for the next stage of development.[21,28]

If we are to use these transcription factors to convert glia cells to neurons. We can think of conversion in two steps. First, a glial cell must overcome cell fate and commit to becoming a neuron. Then the cell must adapt, survive, and mature to become a fully functional neuron. There is concern that constitutive activation of early neuronal transcription factors may prevent the necessary "off switch" to allow for development progression even if the cell can overcome fate commitment. As a result, we would find our cells to be immature and unable to develop further. From this reasoning, we can hypothesize that late stage transcription factors will yield greatest conversion efficiency.

We would expect the order in decreasing efficacy to be NeuroD1, Ngn2, Ascl1, and Sox2.[28]

Adeno-associated viruses (AAVs) are an attractive vehicle for vector delivery because they are non-integrating and non-pathogenic.[22] AAVs can transduce both dividing and nondividing cells with sustained expression rates. In addition, the tropism of AAVs vary among serotypes. Currently there at least 11 different serotypes available. AAV1, AAV2, AAV4, AAV6, AAV8 and AAV9 are the most often used serotypes to deliver genes to the CNS.[27] AAV2 is the most common and has been used in clinical trials. A comparative study reported that AAV8 has the highest specificity for astrocytes in the brain.[28] AAV9 is unique in its ability to cross the blood-brain barrier to infect both glial and neuronal cells.[29] AAV9 exhibits high versatility and high transduction efficiency in and outside of the CNS [29] Additionally, AAV9 and rhAAV10 are reportedly better able to spread in the brain parenchyma and maintain long term expression levels in a diseased brain.[32] Together AAV8, AAV9, and AAV10 show some of the highest *in vivo* transduction efficiencies in the normal CNS but it is unknown if these can be recapitulated in the adult damaged CNS. We wish to directly compare AAV8, AAV9, and AAV10 transduction efficiencies in the injured CNS.

A downside for AAV design is their limited packaging size and promiscuity. Unfortunately, the size of these reprogramming factors are quite large and allow us to only use a single reprogramming factor. The mRNA for Ngn2 is 2.5 kB, Ascl1 is 2.8 kB, Sox2 is 2.5 kB and NeuroD1 is 2.5 kB in size. The large size restricts us to using a single-stranded AAV (ssAAV) with a 4.5 kB capacity instead of the smaller 2.3 kB self-complementary AAV (scAAV) that typically offers higher transduction efficiency.[30]

Previous groups have used retroviruses to target only proliferating glial cells and not post-mitotic neurons. Our proposed AAV system infects both dividing and nondividing cells. This indiscrimination creates two distinct problems. The first problem of non-specific delivery of reprogramming factors may cause our reprogramming efficiency to be diluted when targeting a glial subtype. To target glial cells subpopulation more specifically and efficiently with our reprogramming factors, we will test the effect of two glial specific promoters of GFAP and NG2 that have been previously validated in reprogramming.[33] Reactive astrocytes greatly upregulate glial fibrillary acidic protein (GFAP) compared to their nonreactive counterparts. Post-mitotic neurons do not express GFAP. Neuron-glial antigen 2 (NG2) expression is restricted to oligodendrocyte progenitor cells (NG2 cells), some microglia and macrophages. We anticipate that that astrocytes are more susceptible to reprogramming over NG2+ cells because they in general have greater plasticity.

Astrocytes taken from injured gray matter share similar expression profiles and the ability to form neurospheres as adult neural stem cells.[34,35] Additionally, NF-κB activation in response to inflammation promotes a conversion of astrocytes into a neural progenitor like cell (NPC) with hallmark expression of OCT4, CD44 and MSI-I and downregulated GFAP expression.[31] The ability for astrocytes to dedifferentiate and become more NPC like suggests they may have greater capacity to convert into neurons. However, this same dedifferentiation could make NPC-like astrocytes less responsive to a GFAP specific promoter because they no longer retain high levels of GFAP expression. This poses the question. Are NPC-like astrocytes or GFAP expressing astrocytes the main target for reprogramming cells? If the answer is the former, NPC-like astrocytes

would be less sensitive to a GFAP promoter and thus overall conversion efficiency would decrease compared to a NPC or generic CAG promoter. If the answer is the latter, GFAP expression would be high and the promoter would enhance conversion efficiency. On the other hand, NG2 cells are only known to upregulate NG2 expression in response to inflammation. Thus, a NG2-specific promoter would robustly express reprogramming factor and enhance overall conversion efficiency.

The second problem concerns identification and verification of the new neuron identity. Traditional immunohistochemistry (IHC) would be unable to distinguish between a transduced preexisting neuron and a newly converted neuron. In further studies, we propose bromodeoxyuridine (BrdU) DNA labeling to differentiate between proliferating and non-proliferating populations of cells in the adult brain. Preexisting postmitotic neurons will not incorporate BrdU. However proliferative glial cells that later undergo neuron fate commitment will retain the BrdU label from earlier.

The goal of this study is to directly compare and identify AAV serotype, neurogenic factor and glial subtype that optimize reprogramming efficacy *in vivo*. Past studies have examined each component in isolation and have largely been focused on proof of concept.[21] We wish avoid variability between studies and perform a standardized comparison. The optimized AAV vector developed from our study would be further validated and serve as the foundation for canine stroke model already developed in our laboratory.

Chapter 2. Material and Methods

Overview

The experiment design used a permanent distal middle cerebral stroke occlusion model (MCAO) originally developed for young rats as a platform to measure and compare reprogramming efficacy of adeno associated viruses in young adult mice.[41] This ischemic stroke model was selected because it is reproducible, most closely mimics human stroke, and is relatively non-invasive since it damages only the cortex and not the striatum.[42] More specifically, MCAO induced infarcts have a wide consistent distribution to the frontal, parietal and temporal lobes but are limited in their spread to the thalamus, hypothalamus, hippocampus, and other midbrain structures. On day 1, stroke was induced. On day 5, mice were injected with one of six AAV constructs. AAV-pGFAP, and AAV-pNG2 constructs contained either Ascl1, Ngn2 or neither for a total of six constructs. We waited five days post stroke because proliferation of NG2-glia and reactive astrocytes had occurred by this time point.[11,43] On day 12, mice were sacrificed and perfused. IHC was performed to look for reporter protein (mKate2) and one of the following four markers; reactive astrocyte (GFAP), oligodendrocyte (Olig2), immature neuron (Dcx) or mature neuron (NeuN). Immature or mature neurons labeled with our AAV reporter protein were positive hits for newly reprogrammed neurons.

Our project had a three-fold purpose. First, we wished to identify cells preferentially transduced by AAVrh10. Second, we wished to compare reprogramming efficiency using GFAP and NG2 glial specific promoters. Third, we wished to compare the glial to neuronal reprogramming efficiency of the basic helix loop helix transcription factors Ascl1 and Ngn2.

Animals

All experiments were performed in compliance with animal care guidelines issued by the NIH and by the Institutional Animal Care and Use Committee at the University (IUCAC) of Minnesota. Healthy, wild type, inbred strain C57BL/6J mice were purchased by Jackson Laboratory (Bar Harbor, ME) for the study. Mice were selected between 9 weeks and 10 weeks old to provide the best chance of surviving stroke. They were housed in Research Animal Resource (RAR) specific-pathogen-free (SPF) facilities with a 12-hr light, 12-hr dark cycle with no more than five mice per cage and fed irradiated food with water.

MCAO Stroke Model

The MCAO model was adapted from a protocol originally developed to give permanent stroke for young rats.[41] P60 to 70 C57/B6 mice were anesthetized with Ketamine (Zoetis Inc., Kalamazoo, MI) at 100 mg/kg, and Xylazine (Akorn, Inc., Lake Forest, IL) at 10 mg/kg both given intraperitoneally (IP). Additional doses of ketamine at 50 mg/kg, were administered IP if the animal became responsive to a toe pinch or showed visual signs of discomfort. Alternatively, 1-3% isoflurane with oxygen were to be given intranasally (IN). Body temperature was maintained by an intrarectal thermometer and maintained around 37°C by a heating pad. Animal's head was secured by a headholder (David Kopf Insturments, Tujunga, CA) in a prone position. Fur around the neck and head was shaved and treated with Betadine. Oxytetracycline (Pfizer Inc., New York, NY) ointment was added to protect the eyes. A vertical incision was made behind the right eye to expose the musculature. A sub-fascia dissection was performed to retract muscles to

expose the zygoma. An electric drill (Meisinger USA. LLC, Centennial, Colorado) was used to create a small hole 1-2mm in front of the root of the zygoma to expose the middle cerebral artery. Once exposed the distal middle cerebral artery (MCA) was cut with micro scissors to create a permanent occlusion in the distal MCA and cauterized using 0.25mm tip bipolar cautery (Kirwan, surgical products LLC., Marshfield, MA) The wound was sealed using Prolene sutures (Ethicon US LLC., Somerville, NJ). Mice were given Ketoprofen at 50mg/kg subcutaneously daily for pain and were returned to a heated recovery box. Mice were monitored for three days following surgery for body weight, stress, wound healing and dehydration. In general, mice seemed to display contralateral weakness in their front limbs, asymmetrical movement, and reduced mobility compared to mice who had not yet undergone surgery. If further abnormalities such as labored breathing, shivering, and/or difficulty eating were observed, mice were euthanized with carbon dioxide.

Vector Design

Viral vectors AAV 8, 9 or rh10 were purchased from University of Pennsylvania Vector Core (Philadelphia, PA). Vectors were from stock and made available for general distribution. AAV 8, 9 and Rh10 used to compare general transduction were pre-constructed plasmids composed of CB7 promoter with chicken beta-actin intron (CI), resulting in three recombinant viral vectors (AAV8.CB7.CI.eGFP.WPRE.rBG, AAV9.CB7.CI.eGFP.WPRE.rBG, and AAVrh10.CB7.CI.eGFP.WPRE.rBG). Comparison of conversion efficiency in astrocytes was performed using pre-constructed plasmids composed of Ngn2, Ascl1, empty vector under control of hGfABC1D (GFAP) promoter with an enhanced red fluorescence protein (mKate2) packaged into a AAVrh10

capsid, resulting in three recombinant viral vectors (AAVrh10.hGFfABC1D.hNGN2.mKate2WPRE.hGH, AAVrh10.hGFfABC1D.hASCL1.mKate2WPRE.hGH, AAVrh10.hGfABC1D.mKate2.WPRE.hGH). Comparison of conversion efficiency in oligodendrocyte precursor cells (NG2 cells) was performed using pre-constructed plasmids composed of Ngn2, Ascl1 or empty vector under control of hNG2 promoter with an enhanced red fluorescence protein (mKate2) packaged into a AAVrh10 capsid, resulting in three recombinant viral vectors (AAVrh10.hNG2.hNGN2.mKate2WPRE.hGH, AAVrh10.hNG2.hASCL1.mKate2WPRE.hGH, AAVrh10.hNG2.mKate2.WPRE.hGH)

Administration of AAV

Five days post stroke, AAV ($5x10^{12}$ vg/ml) was injected into the cortex near the area of stroke at two sites. Mice will be injected at a depth of 1.5mm with one of AAV serotypes A 26 gauge needle with 10 ul Hamilton syringe (Hamilton Co., Reno, NV) was loaded with 1 uL at 5×10^{12} GC/ml and injected over 5 min to avoid reflux. The needle was left in the site for 10 min before being retracted. Mice were perfused at 7 days post AAV injection which was also day 12.

Transcardial Perfusion

Immediately following euthanasia, mice were fixed via transcardial perfusion. Mouse was secured in a prone position by taping limbs to a Styrofoam platform in a chemical fume hood. An incision was made along the thoracic midline to the clavicle. The resulting skin flaps were separated and pinned to expose the thoracic field. An

incision was made through the musculature, diaphragm and ribcage to expose the heart. Lastly an incision was made in the right ventricle and a butterfly catheter needle was inserted into the left atrium. An infusion of 10 ml of phosphate buffered saline (PBS) followed by 10 ml of 4% paraformaldehyde (PFA) was performed over 5 minutes or until fluid exiting the right atrium was entirely clear. Mouse brains including olfactory bulbs were removed from the skull and placed in a covered 15 ml vial containing 4% of PFA for 24 hrs followed by 30% sucrose-PBS solution and stored at 4℃. Afterwards, brains were placed in OCT (Fisher HealthCare, Houston, TX), frozen and sectioned at 15 μm using a cryostat. Sections were collected onto charged slides at -30℃. Slides were dried on the benchtop overnight and placed in -20℃ until immunohistochemistry was performed.

Immunohistochemistry

Slides were incubated for 10 min and washed in PBS for 5 min two times. A hydrophobic barrier around sections was created using PAP pen. Cells were permeabilized with J-block (PBS, 1% BSA, 1% Tween-20) for 10 min. Blocking solution (2.5ml Normal Donkey Serum, 1.5ml 10% Triton X-100, 0.05 g Bovine Serum Albumin) was added for 1hour at room temperature. Primary antibody diluted in blocking solution (1:200 to 1:500) was added and sections were stored at 4℃ overnight. Fluorchrome-conjugated secondary antibody diluted in block (1:300 to 1:1000) was added and incubated in a dark room at room temperature for 2 hrs. Sections were washed with PBS three times for 5min each to remove excess primary antibodies. DAPI (1:1000) diluted in PBS was added for 5 min to visualize nuclei. Sections were washed three times for 5 min

to remove excess DAPI. Slides were mounted with immune-mount (Thermo Scientific, Rockford, IL) and sealed with nail polish (New York Color, NYC, NY).

Visualization of Immunohistochemistry

Fluorescent images were acquired using a Leica DMI6000 B microscope (Leica Microsystems Inc., Buffalo Grove, IL) using Leica Application Suite (2012, Build 8587). Cells infected with AAVrh10 were positive with red fluorescence (555 nm ex). Cells stained for neuronal, astrocyte and oligodendrocyte markers displayed green fluorescence (488 nm ex). Nucleus stained with DAPI displayed a blue fluorescence (358 ex). Double positive cells were either preexisting neurons or astrocytes exposed to AAV. Number of double positive and single positive cells were counted in a 20x cell field (~1200 by 900 microns) centered at each AAV injection site. Counts represent an average of 2-4 cell fields over two injection sites. Rabbit polyclonal anti-GFAP antibody (1:500, Dako Agilent, Santa Clara, CA), rabbit monoclonal anti-NeuN antibody (1:500, Abcam, Cambridge, MA) and donkey anti-rabbit Alexa Fluor 488 (1:1000, Thermo Fisher Scientific, Rockford, IL)

Data Analysis

Data shown as mean ± standard deviation of the mean. P-values were calculated using analysis of variance. All calculations were made using the Microsoft Excel program.

Chapter 3. Results

In Vivo Reprogramming of Injured Murine CNS

Previous findings from our lab have suggested that AAVrh10 was statistically more likely to infect astrocytes over neurons ($p<0.05$) (Fig. 1A, B; Dr. Joshua Lim 2016, personal communications). Serotypes AAV9 and AAV8 did not show a significant difference ($p>0.05$). Based on these results, we designed AAVrh10 vectors to better deliver reprogramming factors Ascl1 or Ngn2 to GFAP expressing astrocytes and NG2 expressing oligodendrocyte precursor using glial specific promoters (Fig. 2A). We later set out to compare Ascl1 and Ngn2 glial to neuronal conversion efficiency under control of the hNG2 and hGFAP promoter specific to oligodendrocyte precursors and astrocytes respectively in AAV10 constructs. Three AAVrh10 constructs with a human NG2 promoter and another three constructs with a human GFAP promoter were examined in mice. Stroke was induced on day 1 and constructs were injected near right lateral ventricle with AAVrh10 on day 5. Mice were perfused at 12 days followed by IHC using glial fibrillary acidic protein (GFAP) as a reactive astrocyte marker, doublecortin (DCX) as an immature neuronal marker, neuronal nuclei (NeuN) as a mature neuronal marker, and OLIG2 as an oligodendrocyte precursor marker in green. The red reporter marker mKate2 labelled cells that were both successfully transduced with AAV10 and had either positive GFAP or NG2 expression (Fig 2B).

To determine the specificity of our constructs, we compared the number of double positive mKate2/marker to the total number of mKate2 positive cells (Fig. 2C). In brains infected with vectors containing GFAP specific and NG2 specific promoter, 32 ± 13% and 18 ±10% of mKate2 cells were positive for the GFAP marker, respectively. NG2+

and GFAP+ cells are believed to be discrete and separation populations so we would expect the pNG2 to not target GFAP expressing cells and vice versa.[36] NG2+ glia are known to give rise to GFAP+ positive astrocytes in the injured brain and vice versa; however, these contributions are not considered significant.[37] Instead, the high degree of overlap could be explained as part of an inflammatory response in which microglia become activated and begin to express NG2 and GFAP in response to ischemic stroke.[38,39] Further staining with microglia markers would be needed to support this. Alternatively, the NG2 promoter may be nonspecific and not work well due to its relatively large 1585 base pair (bp) size. Furthermore, GFAP and NG2 promoters showed that 1% and 0% of mKate2+ cells are also positive for the oligodendrocyte differentiation marker OLIG2, respectively (Fig. 2C). Interestingly, the data did not suggest a strong correlation between NG2 promoter activation and an enrichment for the more mature Olig2 marker. Either the promoters do not target the overwhelming majority of NG2+ cells or the cells are not differentiating into Olig2 positive oligodendrocytes in the 7 day time period post stroke. Neither pGFAP nor pNG2 controls were double positive for doublecortin, which is normally associated with neurogenesis in the adult brain (Fig 2D). Lastly, five out of six vectors showed that approximately 10% of mKate2+ cells were also positive for NeuN which was not significantly different than either pGFAP or pNG2 controls ($p>0.05$) (Fig. 2D)

Together these results suggest that overall GFAP and NG2 promoters predominantly express mKate2 in GFAP+ cells but there was a substantial proportion of mKate2 positive cells that were not positive for any of our four cellular markers (Fig 2C). Together GFAP, Dcx, NeuN, and Olig2 positive cells accounted for on average 55% of

all mKate2 cells transduced with AAV10-pGFAP and 30% of all mKate2 cells transduced with AAV10-pNG2 constructs. Additional staining for microglia, pericytes, and endothelial cells will need to be performed to help identify the remaining 50-70% mKate2+ population.

After investigating the general expression profile of our AAV10 vectors, we set out to measure how efficiently our glial-specific promoters could transduce GFAP+ reactive astrocytes and NG2+ oligodendrocyte precursors cells (Fig. 3). Efficiency was calculated by comparing double positive mKate2/GFAP cells to the total number of GFAP cells or by comparing double positive mKate2/OLIG2 cells to the total number of OLIG2 cells. We found that AAV10-pGFAP constructs have a significantly higher expression efficiency of 24 ± 10% for GFAP+ cells compared to their pNG2 counterparts with 7 ± 3% ($p=0.02$) (Fig. 3A). There was no significant difference in OLIG2 expression efficiency between AAV10-pGFAP and AAV10-pNG2 ($p>0.05$). Furthermore, the inclusion of Ascl1 and Ngn2 reprogramming factors did not significantly affect expression efficiency among glial specific promoters ($p>0.05$) (Fig. 3B,C). Our results supported the idea that GFAP promoter enriched for GFAP positive cells while NG2 promoter did not enrich for OLIG2 positive cells. Together, these results suggested that the GFAP promoter would be better suited than the NG2 promoter for delivering the reprogramming factors to the glial population it was designed to target.

Lastly, we examined the glial to neuronal conversion efficiency of neurogenic transcription factors Ascl1 and Ngn2 under control of GFAP and NG2 promoters on day 12 post stroke. Conversion efficiency for induced immature neurons was calculated by number of mKate2/DCX double positive cells to the total number of single positive DCX

cells. We found that only pGFAP and not pNG2 constructs containing the Ascl1 reprogramming factor generated immature neurons significantly compared to pGFAP control ($p=0.04, p>0.05$)(Fig 4A). This result was incongruent with previous findings as Ascl1 alone was known to reprogram both glial cell types into functional neurons *in vivo*.[21] Furthermore, Ascl1 is considered to promote chromatin accessibility but is reliant upon additional network of endogenous pro-neural transcriptional factors for the cell to commit to neuronal fate.[39] Unlike GFAP+ cells which have greater plasticity, the NG2 to neuron transition may be stuck in early developmental stages or require a longer time course to recruit the additional endogenous pro-neural factors needed to commit to the neuronal fate.

We next examined the impact of our AAV constructs in forming mature neurons. Conversion efficiency for induced mature neuron counterpart was calculated by the number of mKate2/NeuN double positive cell to the total number of single positive NeuN cells. AAV10-pNG2 and AAV10-pGFAP lacking the reprogramming factors served as their respective negative controls with a 7 ± 3% and 5 ± 2% background (Fig. 4B). Number of induced mature neurons found using pGFAP-Ngn2, pNG2-Ascl1, and pNG2-Ngn2 were not significantly different compared to control ($p>0.05, p>0.05, p>0.05$). The number of induced mature neurons found using pGFAP-Ascl1 was significantly higher compared to other constructs ($p=0.01$)(Fig. 4A). Ascl1 but not Ngn2 was able to convert GFAP expressing cells past the immature Dcx+ expressing phenotype to the mature Neun+ neuronal phenotype. Our results suggest that GFAP promoter and the Ascl1 reprogramming factor were better able to generate neurons. Overall, the presence of developing neurons and new neuronal growth in the post mitotic adult brain after

injection of pGFAP-Ascl1 pointed towards induction of newly converted neurons. AAV10 has been shown to infect on average 10% of neurons, the inclusion of glial specific promoters should minimize off target neuronal infectivity because GFAP and NG2 are not expressed in neurons.[32] As there is a concern that AAV10 may be transducing preexisting neurons, future experiments will consider incorporating BrdU proliferation assays to differentiate between preexisting and newly formed neurons.

Chapter 4. Discussion

This project is innovative in its push to optimize reprogramming efficiency in an AAV gene delivery system. Currently the field suffers from low efficiency that needs to be greatly amplified before we can see a functional improvement in motor cognition post-stroke. Optimization of reprogramming efficiency is the first necessary step to designing a viable therapy. Previous work has generated a variety of transcription factors, serotypes and sources of cells that all contribute to conversion of glial cells into neurons. Our work performs a three step systematic comparison of findings in the field to solve questions currently unanswered from the past ten years.[21] We first directly compared three serotypes and found that AAV10 is better able to target astrocytes and not neurons (Fig. 1B). We next designed and compared glial specific promoters to predominantly express our reporter protein in GFAP+ reactive astrocytes and NG2+ oligodendrocyte precursor cells. Our results suggest that an AAV10 vector with a pGFAP construct is significantly better able to generate Mkate2 reporter protein in GFAP positive cells compared to its pNG2 counterpart (Fig. 3A). However, a substantial proportion of cells that were double positive for mKate2 reporter protein and GFAP or NG2 expression could not be identified by GFAP, OLIG2, DCX and NeuN cell markers (Fig. 2C). This suggests that

AAV10 vector labels additional cells that could be identified by additional glial or cell markers. Lastly, we compared the reprogramming efficiency of Ascl1 and Ngn2 in NG2+ and GFAP+ cells. We found Ascl1 induced glial to neuron conversion in GFAP+ but not in NG2+ cells (Fig. 4A,B). Previous work has shown that Ngn2 can generate both GABAergic and Glutaminergic neurons while Ascl1 only generates GABAergic neurons from NG2 and GFAP cells.[20,22,23,40] Likewise, we expect Ascl1 to generate GABAergic neurons from GFAP cells in our study. The generation of GABAergic neurons from our model would be considered beneficial in ischemic stroke because they offset the excitoxic effects of glutamate. Shortly after stroke, there is a loss of GABAergic and rise in Glutaminergic neuronal activity that contributes to further neuronal dysregulation and death.[44] By generating GABAergic neurons we can convert glutamate into GABA and minimize excitoxicity that is known to reduce neural degeneration in rodent stroke models.[45] Furthermore, this experiment does not address if induced neurons will properly integrate into nearby circuity. Brain slice electrophysiology experiments will need to be performed to address this question.

In future studies, a GFAP promoter would be the preferred promoter to deliver reprograming factors to reactive astrocytes. However, pGFAP is far from ideal for two major reasons. First, the observed promiscuity of the GFAP promoter is symptomatic of a larger discussion in the field of *in vivo* reprogramming (Fig. 2C). There does not currently exist an ideal selective marker for reactive astrocytes. Other cells appear to upregulate GFAP in response to injury and if we are to design a better promoter, we will need to uncover more selective markers. Second, the coupling of reporter protein mKate2 to GFAP expression leads to underreporting of true reprogramming efficiency values. As

GFAP+ reactive astrocytes transition into neurons, they downregulate both GFAP and mKate2 expression. Consequently, mKate2 signal decays and is lost sometime shortly after reprogramming. This prevents us from differentiating between Mkate2 negative induced neurons and preexisting neurons. Adopting lineage tracing mice or a proliferative incorporating marker like BrdU may alleviate this concern in future experiments by better demarking the astrocyte to neuron transition induced by reprogramming factors. By answering these questions, we hope to ultimately design an AAV construct and begin testing *in vivo* reprogramming in higher order animal models.

A.

AAV8.CB7.CI.eGFP.WPRE.rBG

AAV9.CB7.CI.eGFP.WPRE.rBG

AAVrh10.CB7.CI.eGFP.WPRE.rBG

B.

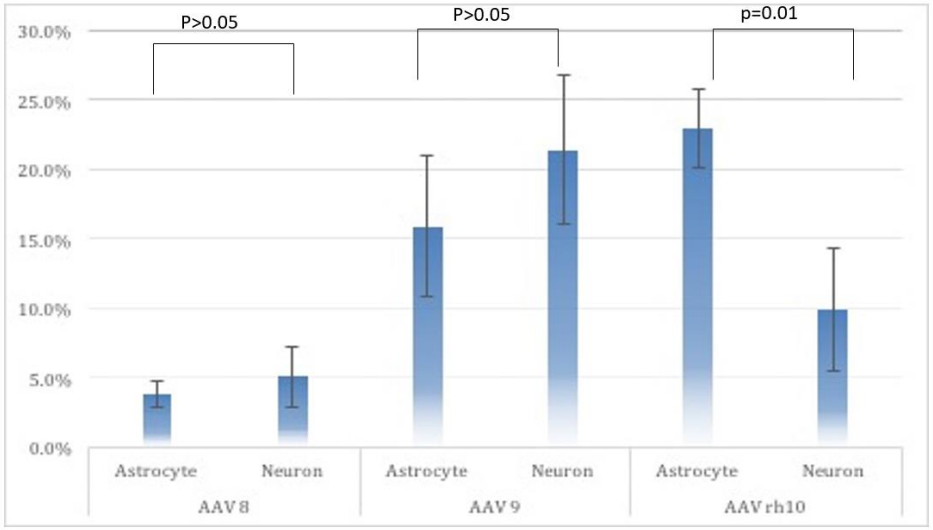


Figure 1. Transduction rates for AAV8, AAV9, AAVrh10 in astrocytes and neurons in adult mouse stroke model. (Dr. Joshua Lim 2016, Personal Communication)

(A) AAV8, AAV9, rhAAV10 are driven by ubiquitous CB7 promoter. (B) Quantification of eGFP/GFAP and eGFP/NeuN double positive cells to identify transduced astrocyte and neurons. eGFP is reporter protein for successful transduction by AAV. Quantification was performed in a 20x field. n = 6 per serotype. mean ± SEM

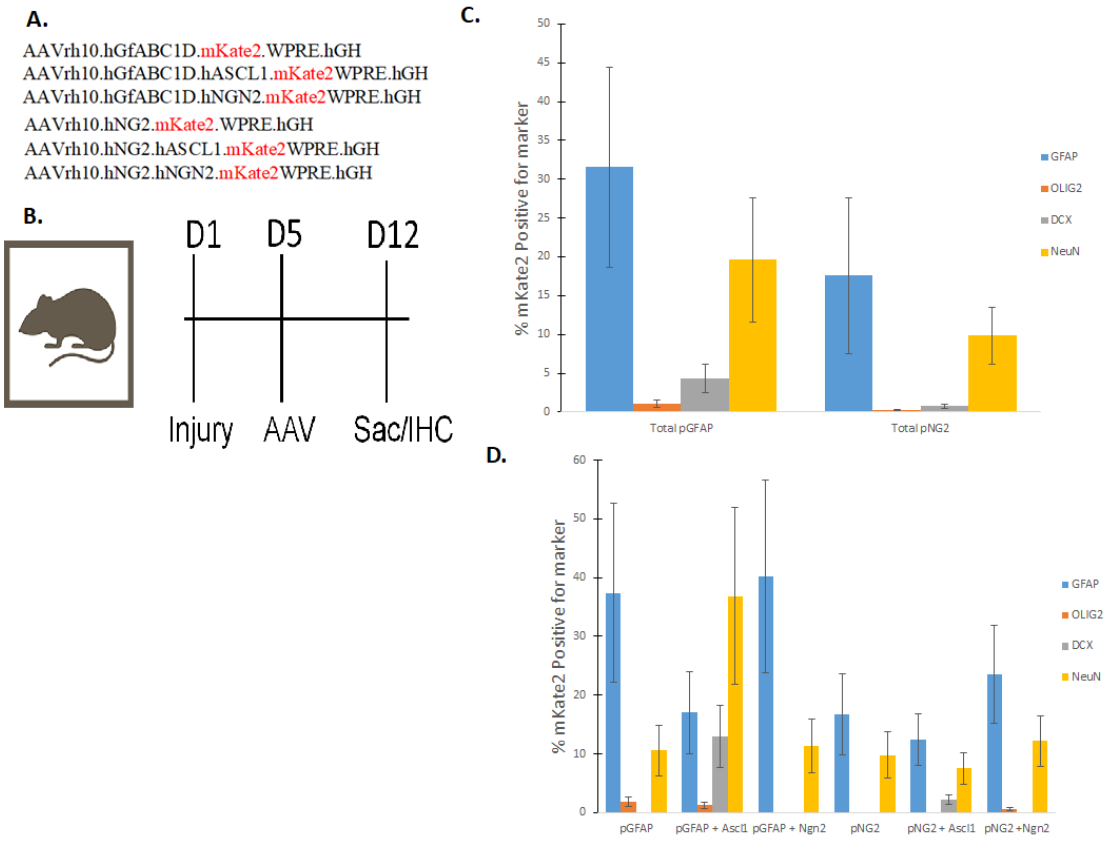


Figure 2. Specificity of AAV10 with hGFAP or hNG2 promoter in adult mouse stroke model

(A) Overview of rhAAV10 vectors packaged with hGFAP and hNG2 promoters (pGFAP,pNG2). Both rhAAV10-pGFAP and rhAAV10-pNG2 contain either hASCL1, hNGN2 or no reprogramming factors (RF). RFs are under control of pNG2 or pGFAP. (B) Timeline of experimental design. (C) Overall rhAAV10-pGFAP and rhAAV10-pNG2 specificity comparing fraction of mKate2+ double cells for respective astrocyte (GFAP), oligodendrocyte precursor (OLIG2), immature neuronal (DCX) and mature neuronal (NeuN) markers to total number of mKate2+ cells. Total pGFAP and pNG2 represent an average of 3 constructs per promoter. Quantification was performed in a 20x field. n = 6, mean ± SEM (D) AAV10 specificity for GFAP, OLIG2, DCX and NEUN for each of the six constructs listed in A. Quantification was performed in a 20x field. n = 6, mean ± SEM

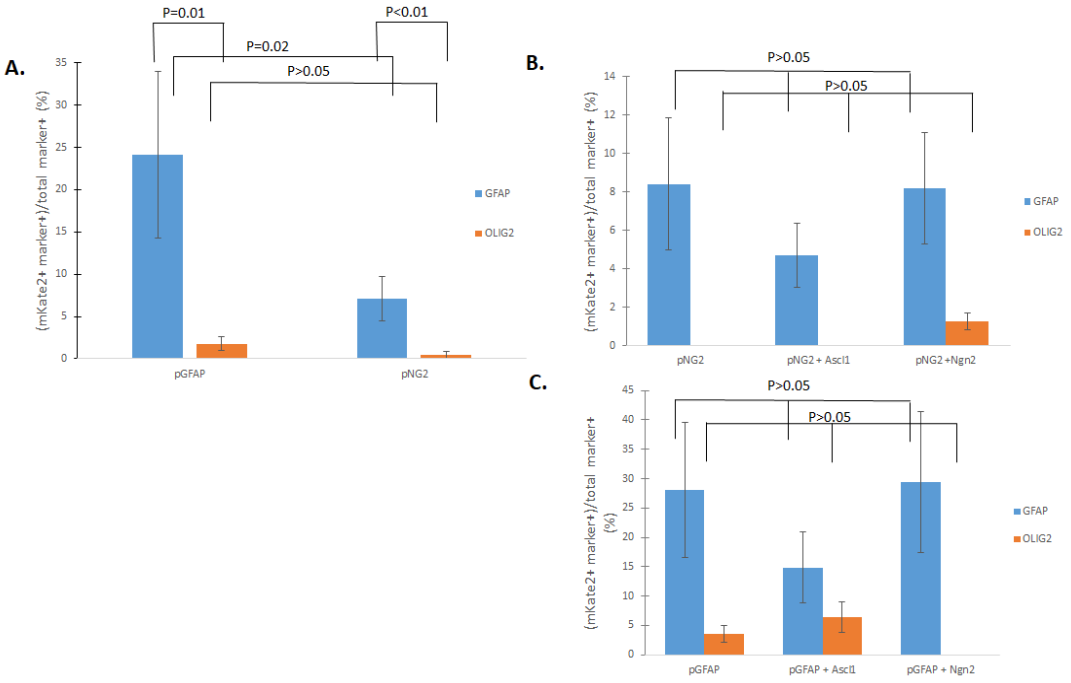
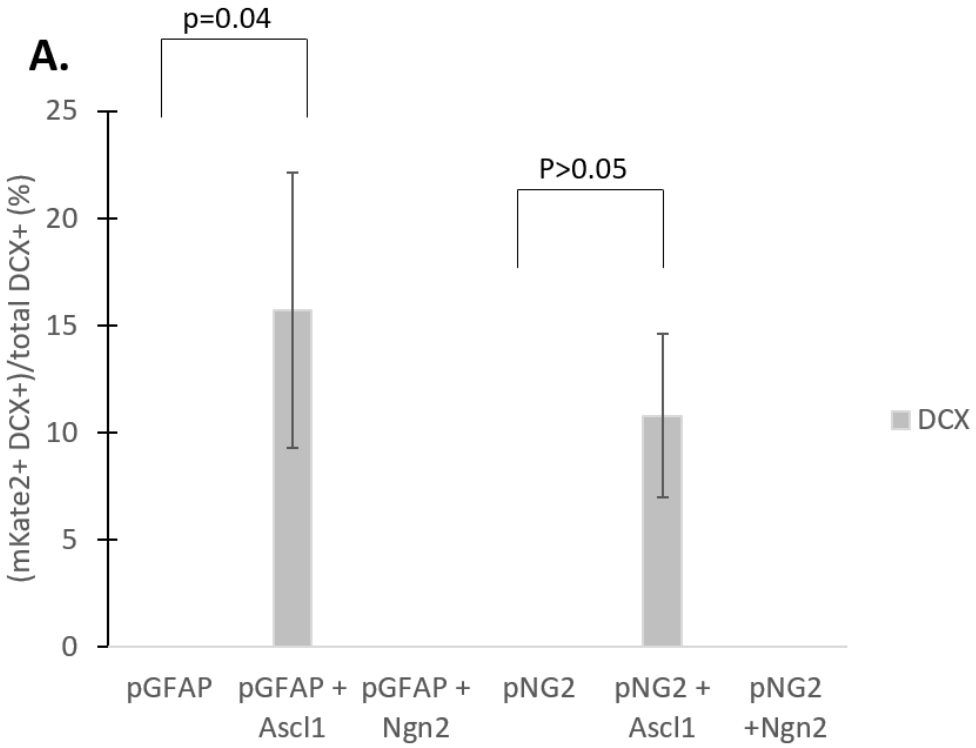


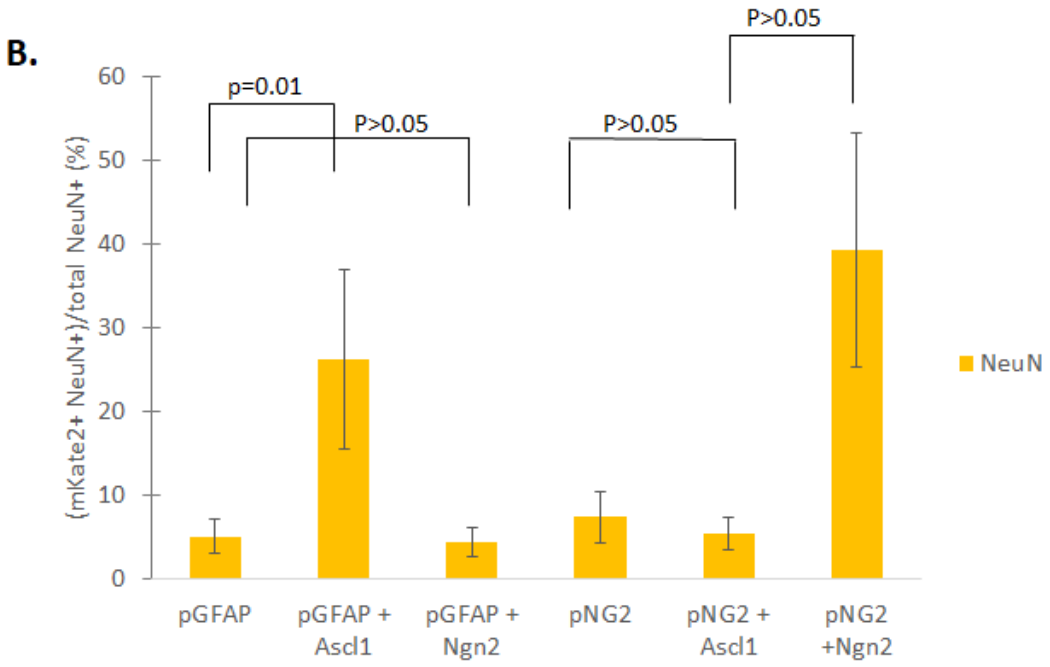
Figure 3. GFAP promoter offers higher expression efficiency over NG2 promoter

(A) Comparison of expression efficiency among all GFAP and NG2 constructs. pGFAP and pNG2 represent an average of 3 constructs per promoter. Quantification was performed in a 20x field. n = 6, mean ± SEM

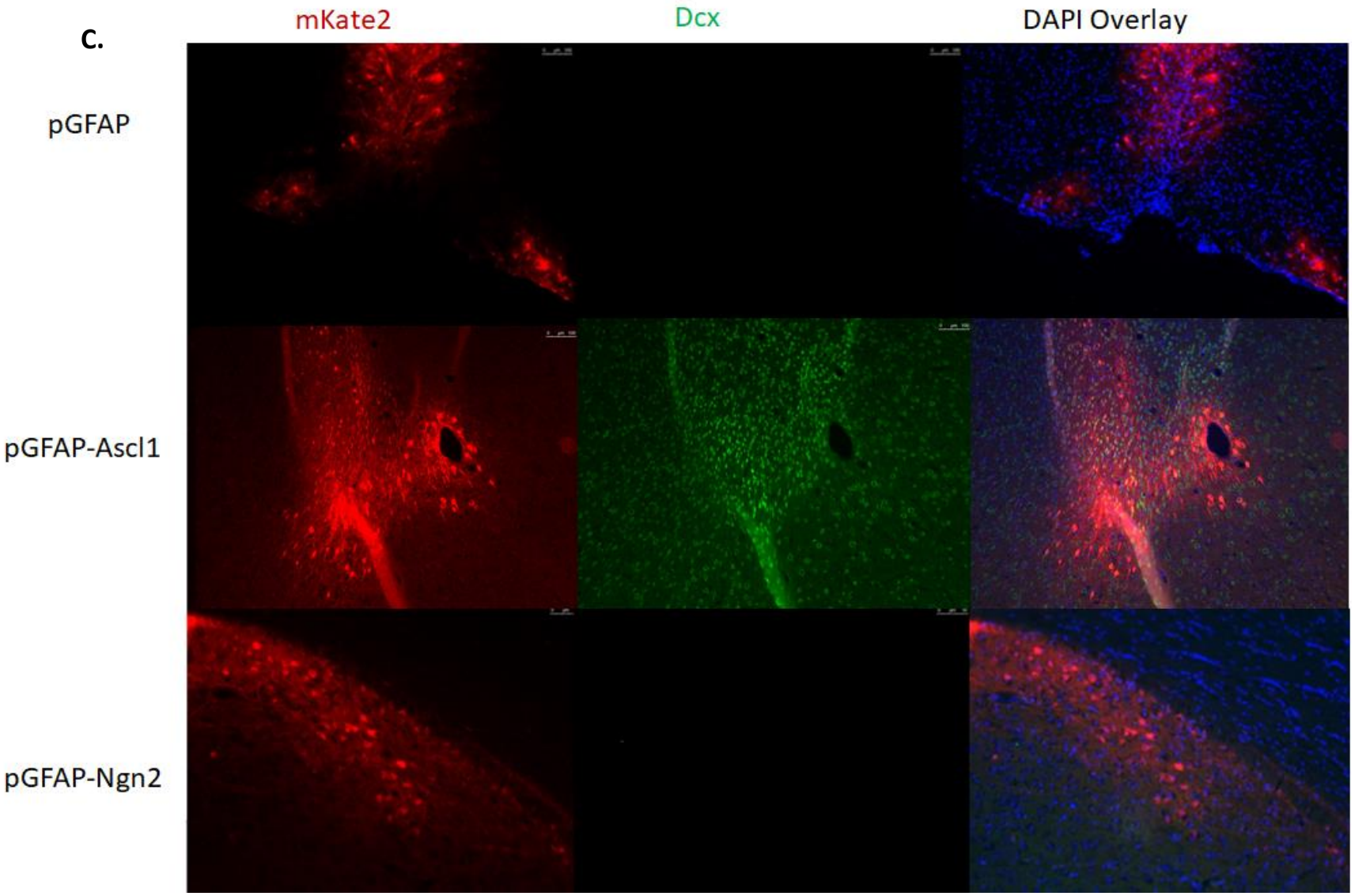
(B) Expression efficiency for GFAP and OLIG2 Positive cells among pGFAP constructs. Quantification was performed in a 20x field. n = 6, mean ± SEM

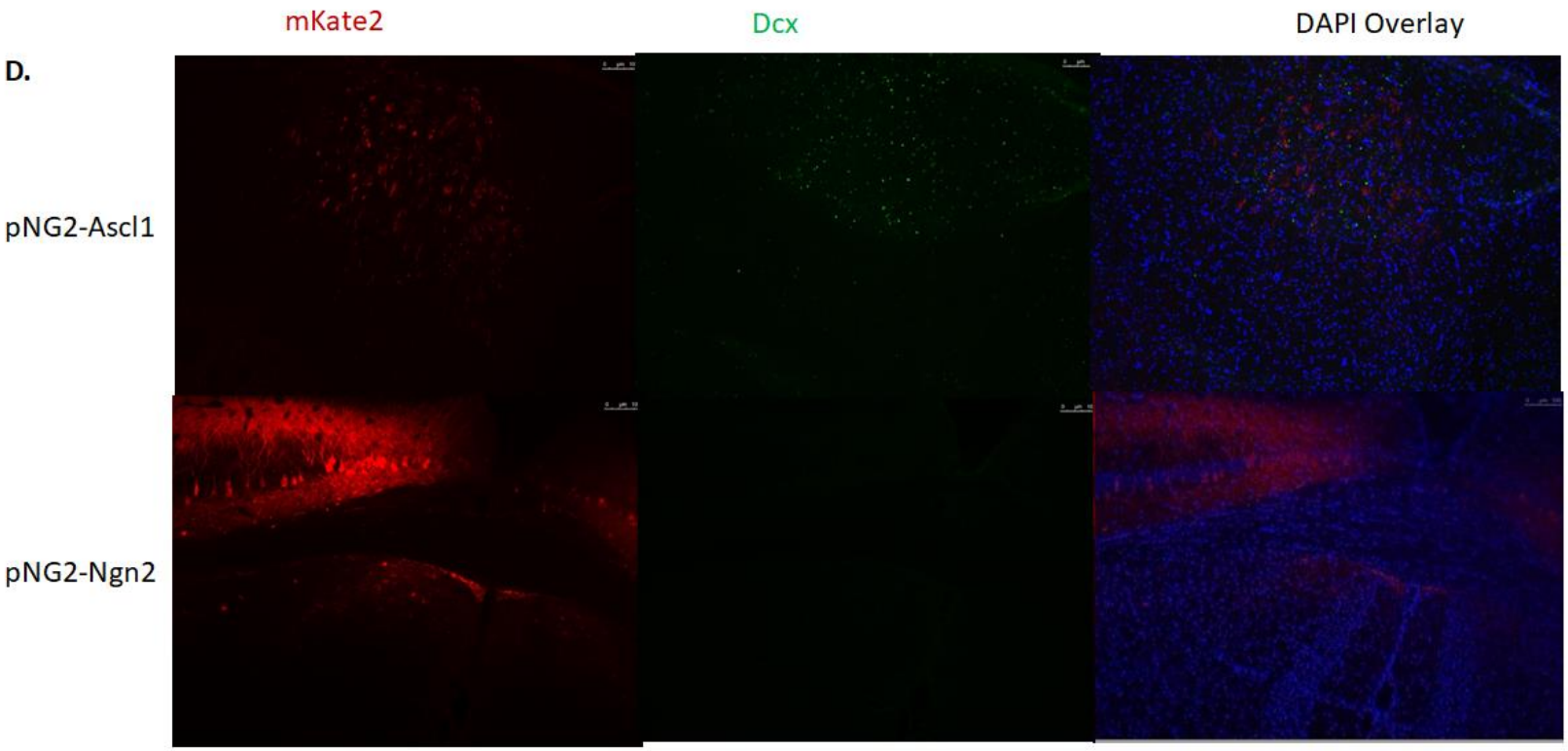
(C) Expression efficiency for GFAP and OLIG2 Positive cells among pNG2 constructs. Quantification was performed in a 20x field. n = 6, mean ± SEM

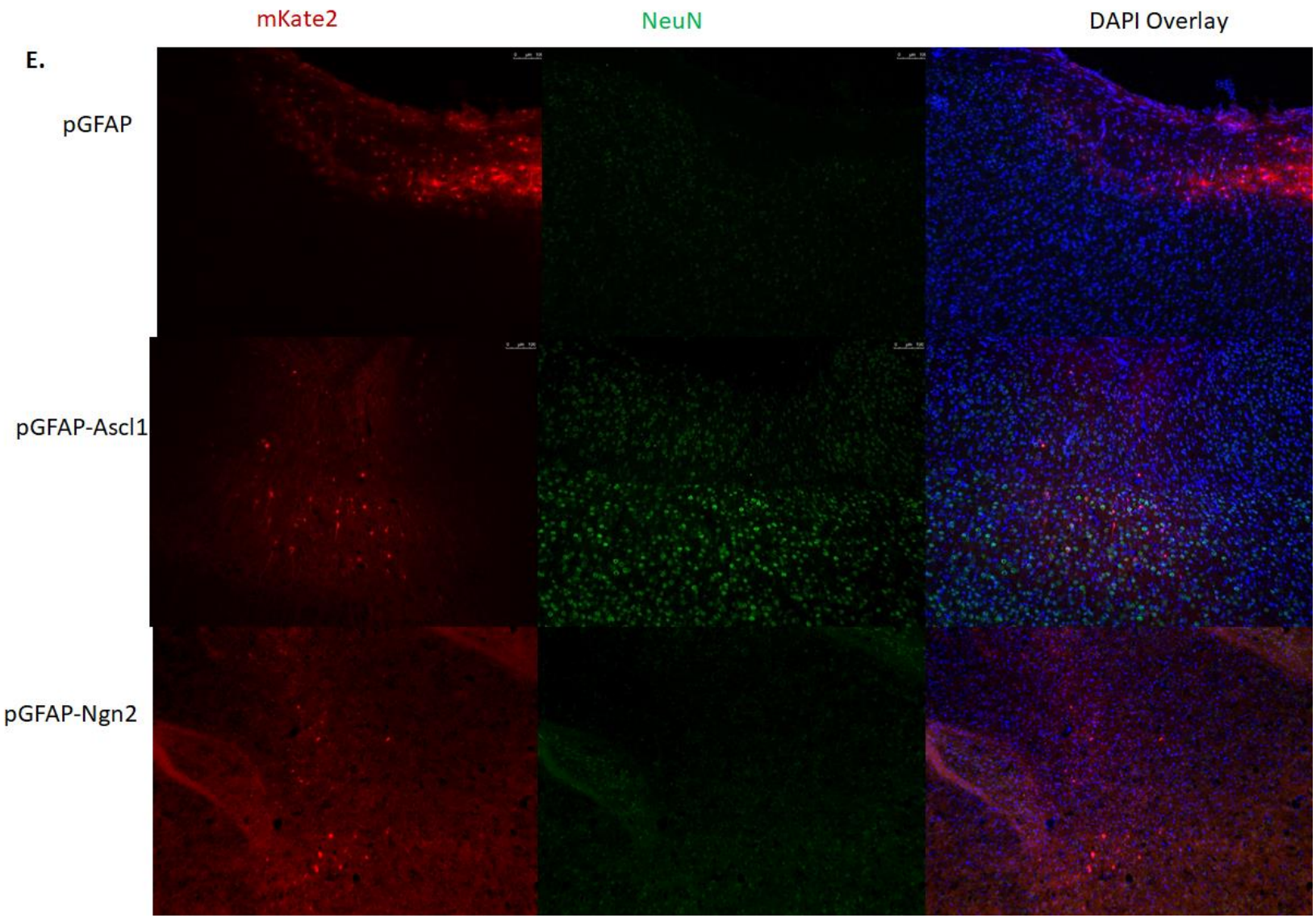

A.
p=0.04
P>0.05
25
20
15
10
5
0
(mKate2+ DCX+)/total DCX+ (%)
DCX
pGFAP
pGFAP + Ascl1
pGFAP + Ngn2
pNG2
pNG2 + Ascl1
pNG2 +Ngn2

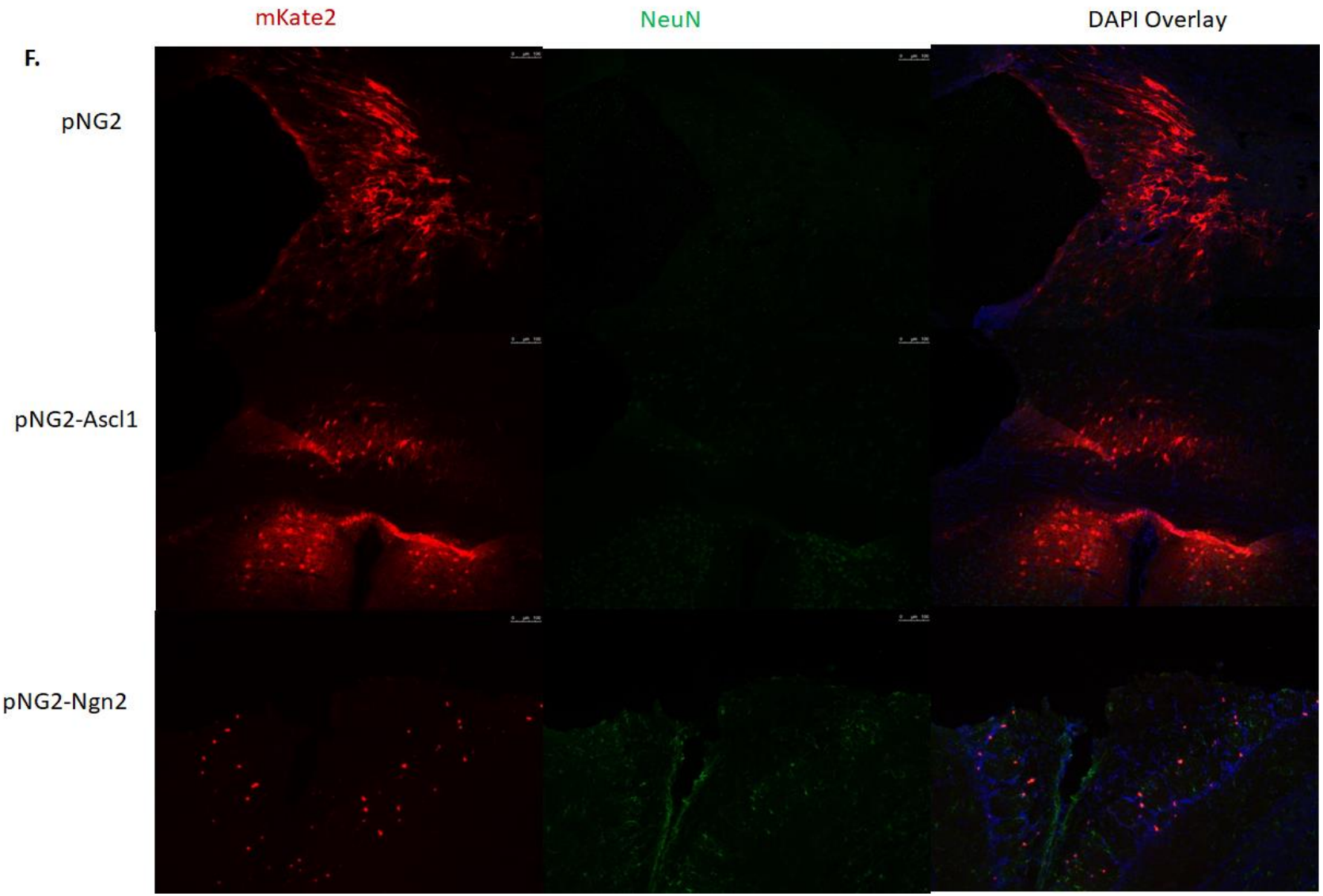

B.
P>0.05
p=0.01
P>0.05
P>0.05
60
50
40
30
20
10
0
(mKate2+ NeuN+)/total NeuN+ (%)
NeuN
pGFAP
pGFAP + Ascl1
pGFAP + Ngn2
pNG2
pNG2 + Ascl1
pNG2 +Ngn2

C.




mKate2
Dcx
DAPI Overlay
D.
pNG2-Ascl1
pNG2-Ngn2


mKate2
NeuN
DAPI Overlay
E.
pGFAP
pGFAP-Ascl1
pGFAP-Ngn2


mKate2
NeuN
DAPI Overlay
F.
pNG2
pNG2-Ascl1
pNG2-Ngn2

G.

	DcX	mKate2	DcX+mKate2+	NeuN	mKate2	NeuN+mKate2+
pGFAP	0 to 0	0 to 54	0 to 0	33 to 117	0 to 105	3 to 13
pGFAP+Ascl1	2 to 86	6 to 97	0 to 21	21 to 204	0 to 81	5 to 43
pGFAP+Ngn2	0 to 7	0 to 87	0 to 0	12 to 91	0 to 64	0 to 9
pNG2	0 to 0	0 to 51	0 to 0	3 to 74	0 to 67	0 to 8
pNG2 +Ascl1	0 to 15	0 to 66	0 to 2	7 to 85	0 to 63	0 to 7
pNG2 + Ngn2	0 to 0	0 to 43	0 to 0	2 to 72	0 to 49	0 to 17

Figure 4. Ascl1 induces glial neuronal conversion in GFAP+ populations.

(A) Quantification showing percentage of mKate2+/Dcx compared to total Dcx cells. n = 6, mean ± SEM

(B) Quantification showing percentage of mKate2+/NeuN compared to total NeuN cells. n = 6, mean ± SEM

(C) Representative images showing pGFAP infected cells co-labeled with mKate2/Dcx at 20x. Scale bar 100 uM. Last row is 40x with 50 uM scale bar

(D) Representative images showing pNG2 infected cells co-labeled with mKate2/Dcx at 20x. Scale bar 100 uM

(E) Representative images showing pGFAP infected cells co-labeled with mKate2/NeuN at 20x. Scale bar 100 uM

(F) Representative images showing pNG2 infected cells co-labeled with mKate2/NeuN at 20x. Scale bar 100 uM

(G) Table displaying the range of cell counts for DcX, NeuN, mKate2 and double positive counterparts.

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